AD_____

Award Number DAMD17-98-1-8241

TITLE: Assessment of mdm2 Alterations on p53 Expression in Breast Cancer

PRINCIPAL INVESTIGATOR: Da-Quing Gao, M.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90033

REPORT DATE: April 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1999	Annual Summary (23 Mar 98 - 22 Mar 99)

4. TITLE AND SUBTITLE
Assessment of mdm2 Alterations on p53 Expression in Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8241

6. AUTHOR(S)
Da-Quing Gao, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
Los Angeles, California 90033

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The mdm2 oncogene regulates the activity of a number of genes critical to genomic integrity. mdm2 can inhibit p53 mediated transactivation as well as control the rapid degradation of p53 protein. mdm2 also inhibits retinoblastoma protein (Rb) and enhances E2F1 and DP1 protein activity. The overall effect is to promote transition to S-phase. This study was undertaken to characterize mdm2 in a cohort of invasive breast cancers and to establish if there are correlations with breast cancer prognostic markers. mdm2 gene amplification was not identified in human breast carcinomas although it has been found in pediatric sarcomas. However, characterization of the mdm2 mRNA by reverse transcriptase-polymerase chain reaction demonstrated not only the expected full-length expression product but alternatively and aberrantly spliced mRNAs as well. A full-length 1526 base-pair mdm2 mRNA was present in 97% of breast cancers analyzed. Smaller mdm2 mRNAs, measuring 653, 281, 254, and/or 219 base-pairs in length, were also identified in 30% of breast cancers. DNA sequence analysis demonstrated that the 653 base-pair mRNAs were the result of alternative splicing while the other smaller mRNAs were the result of aberrant splicing. Each of these alternatively and aberrantly spliced mRNA products was shown to lack important mdm2 functional domains.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer mdm2 gene expression	12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Da-Jo Cobb 4/22/99

Michael O'Brien 4/22/99

PI - Signature Date

ANNUAL REPORT
TABLE OF CONTENTS

Introduction.

The p53 tumor suppressor gene is important in the pathogenesis of many human cancers including breast cancers. Initially, overexpression of the p53 tumor suppressor protein product was associated with mutations in the p53 gene; however, some cancers lacking mutations in p53 have also been shown to have p53 overexpression by immunohistochemistry. This suggests that other proteins in the p53 regulatory pathway may also be important in the pathogenesis of some cancers. In order to test this hypothesis we evaluated the expression of mdm2 in human breast cancer specimens.

The mdm2 oncogene is regulated by p53 and is responsible for down-regulation of p53 activity through inactivation of p53 function and promotion of p53 degradation. mdm2 inhibits p53 function through binding to the transactivation site of p53. After binding to mdm2 p53 is degraded in proteosomes of the cell. The mdm2 oncogene has been described as amplified in some pediatric sarcomas but not in adult cancers. We evaluated mdm2 gene amplification and expression in a series of 38 frozen breast cancer specimens using Southern hybridization of genomic DNA and reverse transcriptase-PCR of mRNA.

Results.

Southern Hybridization. Analysis of genomic DNA from 38 breast cancers and 50 ovarian carcinomas by Southern hybridization showed no amplification of mdm2 in any specimen.

Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR). A nested PCR protocol was used to amplify the full length mdm2 cDNA as described by others with minor modifications as described below. In brief, the full open reading frame of mdm2 (1573 base pairs) was amplified using nested primer pairs. The PCR primers were 5' of the translation start codon and 3' of the translation stop-codon. PCR amplification was performed using 25 µl reactions, containing 1.6 mM $MgCl_2$, 40 pM of each primer, 1 mM dNTPs, 5 units of Taq polymerase (Promega), and approximately 100 ng of cDNA. Thirty cycles of amplification were performed at 94°C (1 minute), 58°C (1 minute), and 72°C (2 minutes) in a Perkin Elmer 480 thermocycler using a

mineral oil overlay. After the external primers were used, a 2 µl aliquot was transferred directly to a new reaction tube with the internal primer pair. Reaction temperatures were the same for both primer sets. The products of the second reaction were run through 1.5% agarose gels (SeaKem LE, FMC Bioproducts, Rockland, ME), stained with ethidium bromide and visualized with ultraviolet transillumination.

RT-PCR analysis of mRNA demonstrated the expected full-length 1526 base-pair (bp) mdm2 product in nearly all breast cancer specimens as well as other, smaller mdm2 products in some breast cancer specimens (Figure 1). Thirty-seven of 38 (97%) breast cancers had the full-length RT-PCR product. In addition, eleven of 37 (30%) breast cancers also had at least one smaller PCR product which measured 653 base-pairs, 281 base-pairs, 254 base-pairs, or 219 base-pairs in length. One sample did not have the 1526 base-pair product but did have a 281 and a 219 base-pair product. Six breast cancers (16%) had both a 1526 base-pair and 653 base-pair products, four breast cancers (10.5%) had 1526 base-pair and 219 base-pair products and one breast cancer (2.5%) had a 1526 base-pair, a 281 base-pair and a 219 base-pair product.

Figure 1

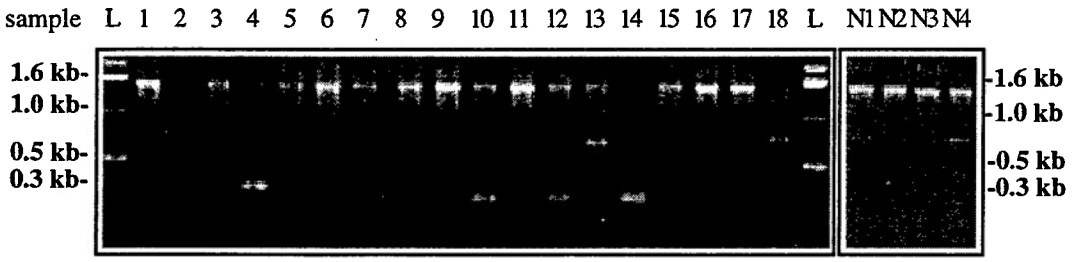


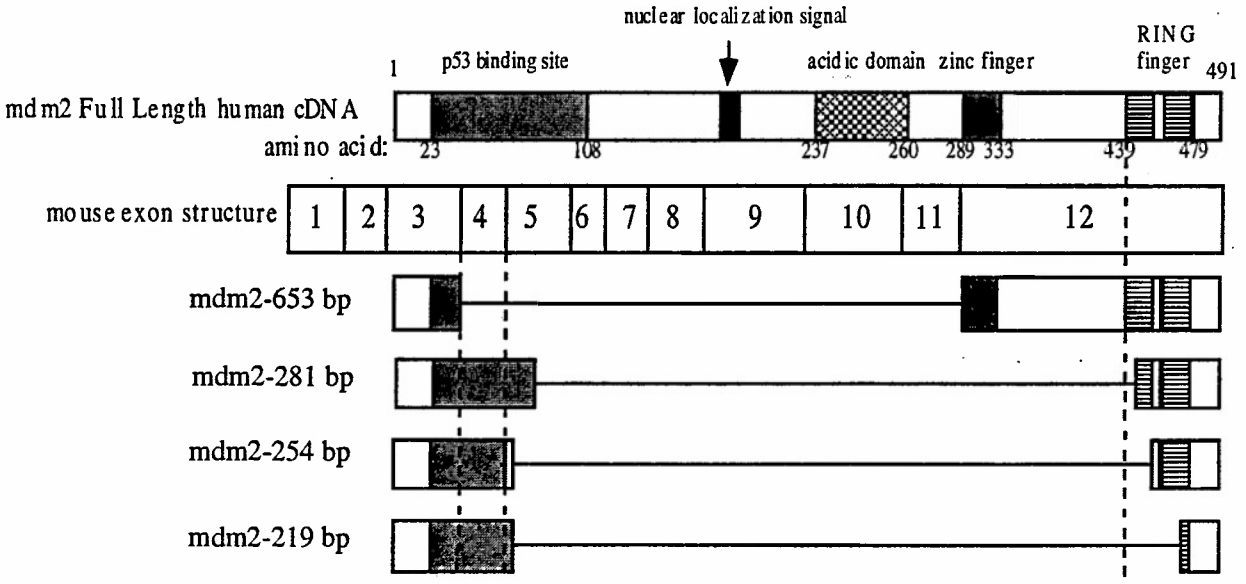
Figure 1. mdm2 alterations in invasive breast cancers and normal breast tissue. An ethidium bromide stained 1% agarose gel shows RT-PCR products derived from invasive breast cancers (1-18) and normal breast epithelium (N). Samples 1-18 are invasive breast cancer; samples labeled N1-N4 are normal breast tissue. "L" indicates the lanes loaded with the 1 kb DNA ladder (Gibco/BRL).

Because of the PCR products less than 1526 base-pairs in length, we decided to evaluate normal breast tissue specimens to determine if any of the smaller products were the result of

alternative splicing of the predicted mRNA. Nine normal breast tissue specimens were analyzed by RT-PCR for mdm2 expression products and all were found to contain the 1526 base-pair product. Four of the nine normal breast specimens also contained a 653 base-pair PCR product (Figure 1). However, the 653 base-pair product was expressed at a much lower level than the 1526 base-pair product.

DNA sequence analysis was performed to determine the identity of the various PCR products (Figure 2). The 1526 base-pair product was confirmed as full-length mdm2. The 653 base-pair product proved to be an alternatively spliced mdm2 in which exon 3 (the exon containing the ATG-start site) was spliced in-frame to exon 12 (the exon containing the termination site). DNA sequence analysis of the other, smaller PCR products confirmed that each contained mdm2 sequences but also demonstrated that each was an aberrant splice variant of mdm2. The aberrant splice sites occurred within exons (Figure 2), not at exon-intron boundaries as is usually observed with alternatively spliced products.

Figure 2. Schematic Comparison of mdm2 with PCR Products of Various Sizes.



A schematic summary is provided comparing the full-length mdm2 cDNA with each of the RT-PCR products which were isolated and sequenced. The functional domains are identified at the top of the figure. (Canvas 5.0)

The 281 base-pair aberrantly spliced mdm2 product consisted of 204 bases of 5' mdm2 open reading frame from exons 3 and 4 spliced to 50 bases from exon 5 and 76 bases from exon 12. The 219 base-pair aberrantly spliced mdm2 product consisted of 155 bases from exons 3 and 4 spliced to the first 16 bases of exon 5 followed by the last 48 bases of exon 12. The pattern of splicing in these mdm2 fragments was of interest because the splice donor and splice acceptor sites were in regions of exact sequence homology within the exons that were spliced.

The 254 base-pair aberrantly spliced mdm2 product consisted of 141 bases from exons 3 and 4 spliced to a 14 base-pair insertion with no know homology, followed by 99 bases from exon 12. The introduction of this 14 base-pair insertion results in a predicted change in the open reading frame.

In each of these small, aberrantly spliced mdm2 products the p53 binding site, the nuclear localization signal, the acidic domain and a portion of the RING finger were all deleted from the mRNA expression product. The loss of these domains is predicted to effect the function of the mdm2 product.

Key Research Accomplishments.

1. Identification of alternative splicing and aberrant splicing of mdm2 in breast cancer.
2. Loss of important functional domain in aberrantly spliced mdm2.

Reportable Outcomes.

None.

Conclusions.

mdm2 mRNA is altered in approximately 30% of human breast cancers. The alteration including loss of functional domains interacting with p53 regulatory domains suggest that mdm2 may play an important role in derangement of the p53 pathway in some breast cancers.

References.

None.

Appendices.

CURRICULUM VITAE

Da-Qing Gao, M.D.

A. Personal Information

Name in Full: Da-Qing Gao

Business Address: Department of Pathology, NOR 5409
Norris Comprehensive Cancer Center
School of Medicine
The University of Southern California
1441 Eastlake Avenue
Los Angeles, California 90033

Business Telephone: (323) 865-0563.
FAX Number: (323) 865-0122.

PII Redacted [redacted]

B. Education

Medical School: Shanghai Medical University (P.R. China), M.D., 1983

Graduate School: Tulane University (New Orleans, LA), MSPH, 1990

C. Professional Background

Senior Fellow, Institute of Parasitic Diseases , Chinese Academy of Preventive Medicine, Section of Biological Parasitology, 1983-1989.

Postdoctoral Fellow, Genetic Pharmacology Unit, Experimental Therapeutic Branch, National Institute of Neurological Disorders and Stroke (NIH), 1990-1991.

Research Associate (Academic Faculty), Department of Pediatric, Georgetown University. Co-lab, Genetic Pharmacology Unit, Experimental Therapeutics Branch, National Institute of Neurological Disorders and Stroke (NIH), 1991-1994.

Postdoctoral Fellow, Neuromuscular Center, University of Southern California, 1994-1994.

Research Associate, Department of Medical Genetics, Cedars-Sinai Medical Center, University of California at Los Angeles, 1995-1996.

Research Associate, Department of Pathology, University of Southern California, 1996-present.

D. Bibliography

1. Zhang JX and Gao D. Advances and the problem of vaccination against malaria (review). Parasitic Diseases Fascicle of Foreign Medicine 4: 151-154, 1987.

2. Liu RJ, Gao D, Liu DQ, Ren DX. On the modification of drug-coated plated to determine the in vitro sensitivity of P .falciparum to pyronariodine and artesunate. Annual Institute of Parasitic Diseases: 86-87, 1987.

3. Zhang LZ, Zhang YM, Wang J, Han M, Feng XP, Chai L, Liu DQ, Gao D. Relationship between malaria infection and ABO blood group and nationality annual of Institute of Parasitic Diseases: 86-87, 1988.

4. Qiu C, Liu DQ, Liu RJ, Gao D, Ren DX. Study on the surveillance of the sensitivity of P. falciparum to pyronaridine and sodium artesunate. Chinese Journal of Preventive Medicine 22(6): 368, 1996.

5. Liu DQ, Ren DX, Liu RJ, Qiu CP, Gao D. A handy medium for in vitro assessment of sensitivity of plasmodium falciparum to chloroquine and melfloquine in the field. Chinese Journal of Parasitology and Parasitic Diseases 7(2): 122-114, 1989.

6. Feng Z, Wan C, Qiu C, Wu Z, Gao D, Liu R. Determination of minimum effective concentration and proposed dosage regiment of pyronridine in the treatment of malaria. Chinese Journal of Parasitology and Parasitic Disease 6(1): 36-38, 1988.

7. Liu D, Liu RJ, Ren DX, Gao, D, Zhang CY, Cai XZ, Ling CF, Liang AH, Tang Y. Alteration in resistance of plasmodium faciparum to chloroquine after cessation of chloroquine medication for twelve years. Chinese Journal of Parasitology and Parasitic Diseases 10: 112-114, 1992.

8. Gao D, Cansesaa L, Mouradian MM, Jose P. Dopamine D2-long receptor gene expression in specific nephron segments demonstrated by reverse transcriptase/polymerase chain reaction. J Soc Nephrol 3: 519, 1992 (abstract).

9. Gao, D, Minwoa T, Jose P, Mouradian MM. Cloning of exon I of the Rat D3 dopamine receptor gene. J Soc Nephrol 3: 511, 1993 (Abstract).

10. Gao D, Minowa T, Jose P, Mouradian MM. Cloning of exon I of the Rat D3 dopamine receptor gene and its expression in renal microvessels. Soc neurosci 19: 1371, 1993 (abstract).

11. Gao D, Canessa LM, Mouradian MM, Jose P. Expression of the D_2 subfamily of dopamine receptor genes in the kidney. American Journal of Physiology 266: F646-F250, 1994.

12. Yamakawa K, Virtaneva K, Gao D, Mitchell S, Lyons G, Kim U, Lehesjoki AE, de la Chapelle A, Korenberg JR. EHOC-1: A candidate gene for progressive myoclonus epilepsy on 21q22.3. American J of Hum Genet 57:4 A154, 1995 (abstract).

13. Wang W, Hahn K, Bishop J, Gao D, Jose P, Mouradian MM. Up-regulation of D_3 dopamine receptor mRNA by neuroleptics (submitted, 1995).

14. Wang W, Hahn K, Bishop J, Gao D, Jose P, Mouradian MM. Up-regulation of D_3 dopamine receptor mRNA by neuroleptics. Society for Neuroscience 21(2): 853, 1995 (Abstract).

15. Yamakawa K, Gao D, Korenberg J. A periodic tryptophan protein 2 (PWP2) gene homologue maps in the candidate region of progressive myoclonus epilepsy on 21q22.3 (submitted, 1995).

16. Yamakawa K, Virtaneva K, D'Amato E, Gao D, Huo Y, Fannin S, de la Chapelle A, Lehesjoki A, Korenberg J. Exon-intron organization and splicing intermediates of the EHOC-1 gene and mutational analyses in EPMI patients (submitted, 1996).

17. Yamakawa K, Gao D, Korenberg JR. A periodic trptophan protein 2 gene homologue (PWP2H) in the candidate region of progressive myoclonus epilepsy on 21q22.3. Cytogenet Cell Genet 74: 140-145, 1996.